ENZYMATIC DE[illegible]N BY LACCASES

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ABSTRACT

More than half of platform petrochemicals are aromatic, whereas the only large-scale, naturally-occurring, renewable source of aromatics is lignin. Chemical depolymerization of lignin requires extreme conditions, and results in extensive destruction of the aromatic rings and/or char formation. By contrast, enzymatic lignin depolymerization occurs under mild conditions with retention of the aromatic nuclei. Therefore, laccase from *Agaricus bisporus* (LAB) and from *Trametes versicolor* (LTV) with the mediator, ABTS (2,2'-azino-bis(3 ethyl benzthiazoline-6-sulphonic acid)) were used to depolymerize lignin (sodium lignosulphonate) under mild reaction conditions with the aim to obtain high concentrations of value-added chemicals. The depolymerization in the presence of LTV was higher than LAB, which resulted from the high catalytic activity of LTV. Lignin degradation resulted in formation of complex product mixtures. Therefore the products were fractionated and analyzed by different analytical techniques including GPC (for preliminary screening), HPLC and GCMS (for product characterization and quantification), and NMR (for fingerprint analysis). Products included guaiacol, vanillin, acetovanillone, vanillic acid, homovanillyl alcohol, phenol, 4- methylbenzaldehyde, catechol, *p*-toluic acid, 4-hydroxybenzaldehyde, tyrosol, isovanillin, and 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) propan-1-one, and the total yield of monomers from lignin was 9.8 % in the presence of LTV. The parameters involved in the depolymerization process were optimized to increase the yield of monomers. The efficiency of laccase mediators was also explored by the use of 2,2,6,6-tetramethylpiperidin-1-yloxy (TEMPO), 1-hydroxybenzotriazole (HBT), *N*-hydroxyphthalimide (HPI) and violuric acid (VLA) in the depolymerization of sodium lignosulphonate. However, the catalytic depolymerization in the presence of these mediators was lower than ABTS. In order to improve the solubility of the substrate for the depolymerization process, screening of ionic liquids that are compatible with LAB was deployed in order to find laccase-friendly ionic liquids for further use in lignin depolymerization. The study has found [C_4mim] [L-tartrate] as the best ionic liquid tested, that increased the activity of LAB by 90 %. In conclusion, enzymatic depolymerization of lignin offers a greener process than the chemical methods, and also provides a more efficient method to obtain monomers of valuable specialty chemicals under mild reaction conditions.

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LIST OF ABBREVIATIONS

Time, length, weight, volume and concentration:

h	hours
min	minutes
s	seconds
n	nanometres
μm	micrometres
g	gram
mg	milligram
μl	microliters
ml	milliliters
L	litres
mM	milimolar
M	molar

General abbreviations:

A	Absorbance
ACN	Acetonitrile
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BSTFA	bistrimethylsilyltrifluroacetamide
°C	degree celcius
DCM	dichloromethane
DMSO-d_6	deutrated dimethyl sulfoxide
EA	elementary analysis
EI	electron ionization
ET	electron transfer
EPR	electron paramagnetic resonance
GC	gas chromatography
GCMS	gas chromatography mass spectroscopy
GPC	gel permeation chromatograpgy

HAT	hydrogen atom transfer
HBT	hydroxybenzotriazole
HPI	*N*- hydroxyphthalimide
HPLC	high performance liquid chromatography
K_m	value of substrate concentration at 1/2 V_{max}
L	light path length
LiP	lignin peroxidase
LAB	laccase from *Agaricus bisporus*
LTV	laccase from *Trametes versicolor*
LMS	laccase mediator system
mV	millivolts
MnP	manganese peroxidase
MeOH	methanol
mol	moles
NMR	nuclear magnetic resonance
OD	optical density
ppm	part per million
[P]	product concentration
rpm	round per minute
RI	refractive index
SD	standard deviation
SEC	size exclusion chromatography
SEM	screening electron microscope
[S]	substrate concentration
[S_o]	initial substrate concentration
t	time
THF	tetrahydrofuran
TMCS	trimethylchlorosilane
TEMPO	2,2,6,6-tetramethylpiperidin-1-yloxy
uv	ultra violet
v_o	initial rate of reaction

V	rate of reation
VOC	volatile organic compound
VLA	violuric acid
V_{max}	maximum velocity
v/v	volume per volume
w/w	weight per weight
w/v	weight per volume
λ	wavelength
ε	extinction coefficient

Chapter 1

AIM AND SCOPE OF THE THESIS

The aim and scope of the thesis is to explore the depolymerization of sodium lignosulphonate to value-added chemicals by an enzymatic process. In this study, commercial laccase was used since the isolation of laccase from lignin-degrading microorganisms such as white rot fungi are generally slow growing and may be difficult to cultivate at scale. In addition, laccase is produced on a large scale due to the widespread applications in biotechnology including in paper manufacturing, detergent formulations, bioremediation, biotransformation, lignocellulose processing, *etc.* (Yaver *et al.*, 2001). Therefore, a commercially available laccase was preferred. There are several factors that contribute towards the efficiency of the enzymatic conversion of lignin by laccase, which is complex from the chemical and biological points of view. A process was developed to study the effect of laccase from *Agaricus bisporus* (LAB) on the degradation of sodium lignosulphonate in the presence of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as a mediator. After the enzymatic depolymerization by laccase, a complex mixture of the products was formed, that is extremely difficult to analyze. Therefore, a fractionation method was implemented to simplify the analysis process and a combination of analytical methods was deployed to identify the products.

Several studies have implemented size exclusion chromatography (SEC) to study the molecular weight distribution of the products (Majcherczyk and Huttermann, 1997; Nugroho *et al.*, 2010; Shleev *et al.*, 2006). Gel permeation chromatography (GPC) is a type of SEC that separates on the basis of size. Bourbonnais *et al.* (1995) have

demonstrated the use of GPC to analyze the oxidation of Kraft lignin by laccase from *Trametes versicolor*. From their observation, the process produced molecular weight averages between 7800 to 10500 $gmol^{-1}$ after several days of treatment. In this project, the aim would be to produce compounds that have a molecular weight below 1000 $gmol^{-1}$. Therefore, GPC was adopted as a part of the preliminary screening of the product distribution after fractionation.

Other than GPC, proton nuclear magnetic resonance (^{1}H-NMR) was implemented to provide chemical information about the products. In ^{1}H-NMR, a chemical shift is associated with the occurrence of various types of chemical resonance present in the sample. Therefore, this technique was used as a fingerprint analysis of the products. NMR analysis has become one of the important milestones for lignin chemistry. However, it has to be noted that the characterization of lignin depolymerization products is difficult due to the complex mixture of products and overlapping signals.

Therefore, gas chromatography mass spectroscopy (GCMS) was also employed to characterize the products. Pecina *et al.* (1986) demonstrated the use of GCMS for the analysis of lignin degradation products. In their work, a method of derivatization was implemented to increase the volatility as well as the detectability of the products. However, derivatization is not always necessary for GCMS unless the compound of interest cannot be detected. In addition, quantification by GCMS was carried out by measuring the peak area of individual components and comparing with authentic standards. Besides GCMS, high performance liquid chromatography (HPLC) has been used for quantification in several studies (Pecina *et al.*, 1986; Bourbonnais and Paice, 1990; Bourbonnais *et al.*, 1997; Vigneault *et al.*, 2007). Thus, an attempt was made to develop an analytical method by using reversed-phase high performance liquid chromatography (RP-HPLC) for the quantification of lignin depolymerization products in conjunction with GCMS analysis. In this study, the identification of the products by GCMS revealed five compounds formed after the enzymatic depolymerization by LAB. However, the yield was only 7.8 % of the total lignin used.

Therefore, the next aim was to further increase the product yield by using laccase from a different source, to influence the efficiency of product formation from the breakdown of sodium lignosulphonate. Therefore, laccase from *Trametes versicolor* (LTV) was studied. The optimum reaction condition in the presence of LTV was explored, with respect to the reaction time, temperature and also the stability of LTV during the course of the reaction.

Even though ABTS is known as the best mediator for laccase (Morozova *et al.*, 2007; Bourbonnais and Paice, 1992), there are more than 100 possible mediators which have been classified into two types, namely natural and synthetic mediators (Canas and Camarero, 2010). Since the synthetic mediators have been proven to be the most effective mediators by several authors (d'Acunzo *et al.*, 2002; Fabbrini *et al.*, 2002), a study on the effect of five synthetic mediators on lignin depolymerization was implemented. Despite the addition of mediators into the reaction however, there is a major drawback since they are expensive (Li *et al.*, 1999; Couto *et al.*, 2005). Therefore, the process for lignin depolymerization by LTV was designed to use the least amount of mediator as possible.

Laccase has a variety of applications. In some cases however, the processes are inefficient because the substrate is insoluble in water. Therefore, it would be desirable to identify enzyme-friendly solvents that can be used to solubilize the substrates. Ionic liquids are a relatively new type of non-aqueous solvent which often perform better in biocatalytic processes than conventional solvents (Cull *et al.*, 2000). Most importantly, there are millions of ionic liquids, offering a variety of chemical and physical properties. This allows the structure of the ionic liquids to be fine tuned to match the specific requirements of the desired process. Therefore, 106 ionic liquids were tested for their effect on laccase from *Agaricus bisporus* (LAB) using a new high throughput screening method (Rehmann *et al.*, 2012). 2,2'-Azino-*bis*(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was used as the substrate, and the Michaelis-Menten kinetic parameters were determined.

Chapter 2

LITERATURE REVIEW

2.1 The Need for Lignin Utilization

Petroleum feedstocks are used in industry to produce a variety of products including fine chemicals, *etc.* (Bender, 2000; Demirbas, 2005). As reported by the Association for the Study of Peak Oil and gas (ASPO), the production of petroleum will decline gradually every year starting from 2010 (Fig. 2.1). The decreasing supply of this feedstock has forced the need to find new alternatives to meet the high demands of value-added chemicals in various applications.

In a sense, the fossil fuels are a one-time gift that lifted us up from subsistence agriculture and eventually should lead us to a future based on renewable resources - Kenneth Deffeyes (2001).

Therefore, an alternative approach was explored based on the potential of lignin as a renewable feedstock for the production of valuable aromatic chemicals that are usually derived from petroleum. According to Gargulak and Lebo (2000), there is an estimated 50 million tonnes of lignin available per year from pulping processes worldwide and only 2 % is in use for commercial applications (Gargulak and Lebo, 2000).

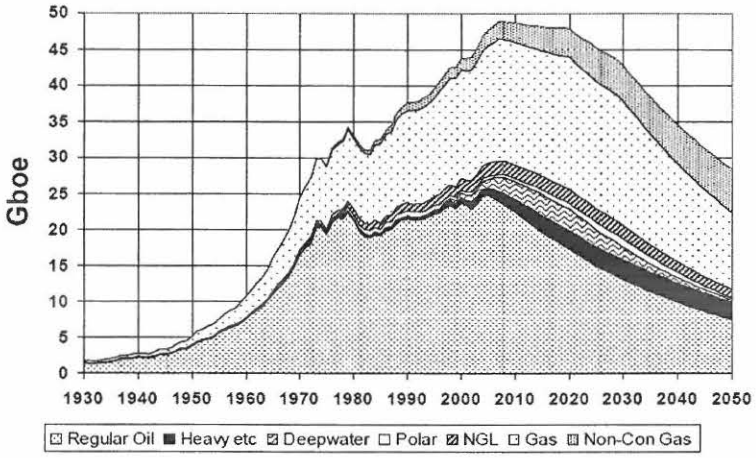

Figure 2.1 Oil and gas production profiles around the globe in 2008 taken from ASPO newsletter No. 100, April 2009. Gboe represents a giga barrel oil equivalent (ASPO, 2009).

2.2 Lignocellulose and lignin

The past decade has seen the rapid development of lignocellulosic biomass as a sustainable source of sugars for biotransformation into biofuels and valuable chemicals (Li *et al.*, 2008; Himmel *et al.*, 2007) especially in the fibre, paper, membrane, polymer and paint industries (Swatloski *et al.*, 2002). Lignocellulosic materials consist mainly of complex structures of the carbohydrates, cellulose (35-50%) and hemicelluloses (20 – 35 %), and lignin (5 - 30 %), a polyphenolic structure (Lnyd *et al.*, 2002; Zavrel *et al.*, 2009; Fig. 2.2).

Cellulose and hemicelluloses are easy to hydrolyze to their subunits (*e.g.* glucose, fructose, galactose, mannose, xylose). The transformations of celluloses and hemicelluloses to the monomer units are a relatively simple process. Numerous studies have attempted to obtain the conversion of cellulose to other products such as bioethanol as a promising alternative energy source to replace crude oil that is likely to suffer limited availability (Demirbas, 2005; Sun and Cheng, 2002). In 2009, Buckeye Technologies, Inc in association with Myriant and University of Florida have announced the development of a new generation bioethanol plant from cellulose which was believed

to be a step forward towards new source of fuel from renewable feedstock (Buckeye Technologies Inc., 2009).

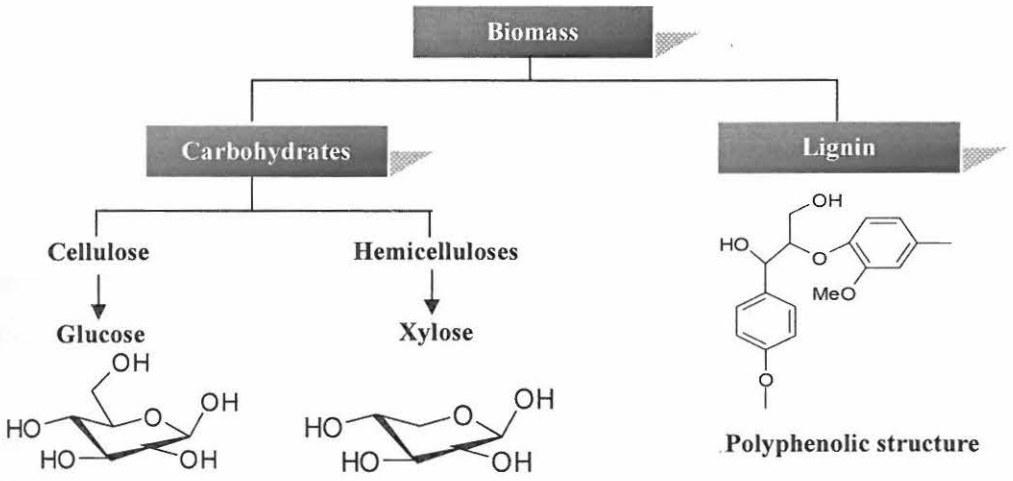

Figure 2.2 Components of lignocellulosic biomass taken from Rogers *et al.* (2002).

On the other hand, lignin is a polyphenolic material composed of phenylpropane units (Rogers *et al.*, 2002). Lignin is practically impossible to dissolve in water in its native form due to the irregular three dimensional cross-linked networks that bind the whole wood structure together to make a strong and resistant plant wood (Kilpelainen *et al.*, 2007). It may also play an important role in defence against pathogen attack and mechanical wounding (Hawkins *et al.*, 1997). The toughness of a plant depends on the percentage of lignin in the cell wall structure. For example, hardwood plants (Fig. 2.3a) contain more lignin compared to softwood plants (Fig. 2.3b) (Antai and Crawford, 1981).

The first serious discussion and analyses of lignin emerged in 1838 in a study by Anselme Payen (Frenh, 2000). He treated wood with nitric acid to remove part of the wood substances and left behind fibrous materials which he called 'cellulose'. He realized that the part that had been removed from the wood materials was rich in carbon content compared to the cellulose. He called the carbon-rich substance as an 'encrusting material' (French, 2000).

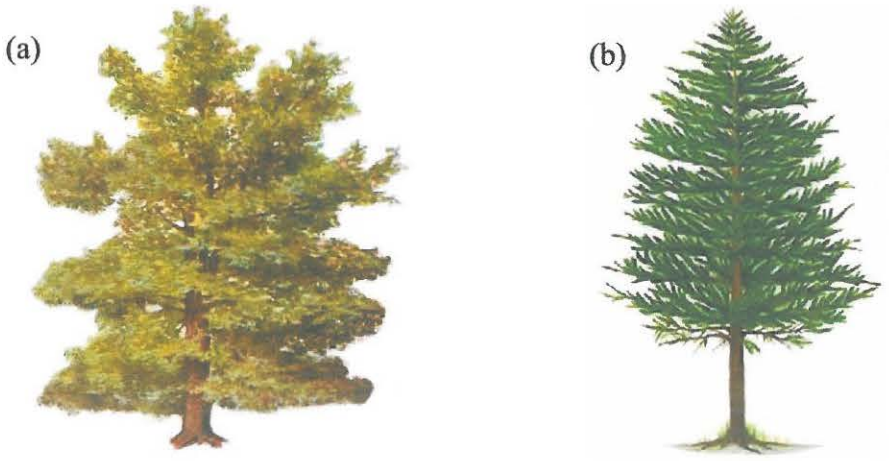

Figure 2.3 Woody plant (a): Hardwood plant (beech tree); (b): Softwood plant (pine tree) (taken from Karen whimsy, 2013 and Peacock river ranch, 2012)

Over the past 100 years, research into lignin has developed and enlarged beginning with work by Schulze in 1865 who first introduced the term 'lignin' (Lu and Ralph, 2010). Three years later, Erdmann in 1868 concluded that the non-cellulosic constituent in wood substances was aromatic. Further investigation of lignin was then demonstrated by Benedikt and Bamberger in 1890 in which they found that methoxyl groups were present in wood tissue but such tissues were lacking in cellulose materials (Brunow, 2001). Further research was done by Klason who came up with the idea in 1897 that lignin was chemically related to coniferyl alcohol (Sjöström, 1993).

Lignin is the second most abundant polymer in nature after cellulose (Annele, 1994; Leonowicz *et al.*, 1999; Li *et al.*, 2008; Zavrel *et al.*, 2009; Kilpelainen *et al.*, 2007; Adler, 1977). It is classified into three major groups which are softwood lignin, hardwood lignin and grass lignin based on the chemical structure of the monomer units (Adler, 1977) which build to form an aromatic, 3-dimensional and amorphous structure (Brown, 1985). Lignin is built from three precursors which are *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol as shown in Figure 2.4 and these precursors are incorporated in lignin as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), respectively (Grabber *et al.*, 1997).

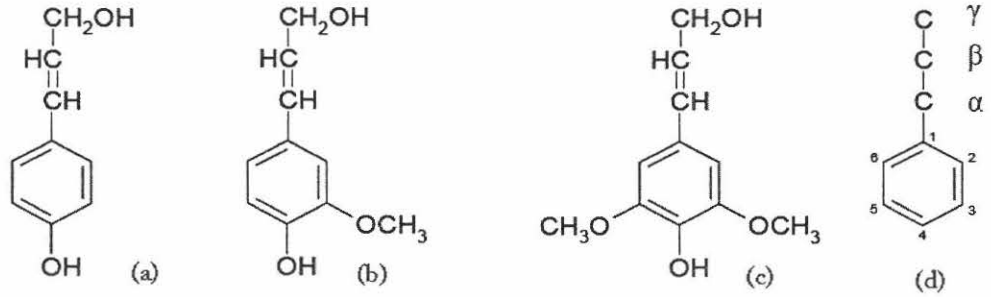

Figure 2.4 Lignin precursors (a) *p*-coumaryl alcohol **(H)**; (b) coniferyl alcohol **(G)**; (c) sinapyl alcohol **(S)**; (d) model for numeration of a carbon skeleton which consist of the aromatic nucleus and 3-carbon side chain represented by γ, β and α (taken from Buswell and Odier, 1987).

These precursors then form different types of subunits of lignin macromolecules where the most abundant subunit is the guaiacylglycerol-β-aryl ether (β-O-4) substructure (40-60 %) followed by biphenyl and dibenzodioxocin, 5-5 (18 – 25 %), phenylcoumaran, β-5 (9 – 12 %), 1,2-diaryl propane, β-1 (7 – 10 %), phenylpropane α-aryl ether, α-O-4 (6 – 8 %), diaryl ether, 4-O-5 (4 -8 %) and β-β linked structures (Adler, 1977; Higuchi, 1990; Sakakibara, 1983; Fig. 2.5) *etc*. A large and growing body of literature has shown that there are no single repeating bonds between the subunits, but a random distribution of at least ten types of bonds (Argyropoulos and Menachen, 1997). The β-aryl ether (β-O-4) bond was the most common bond found in lignin molecule as shown in Fig. 2.5 (Buswell and Odier, 1987). The bonds in lignin are complicated and non hydrolysable, and are much more difficult to break down compared to cellulose and hemicelluloses that are just made from a simple structures and linked with β-1, 4-glucosidic bonds (Kuhad *et al*., 1997).

Lignin has a high molecular weight which makes it a tough structure and prevents its uptake into the microbial cells (Eriksson *et al*., 1990). Due to this fact, biological degradation of native lignin must occur through the activity of extracellular enzymes (Adler, 1977; Argyropoulos and Menachen, 1997; Kuhad *et al*., 1997; Eriksson *et al*., 1990) from lignin degrading microorganisms such as white rot fungi (Hatakka, 1994; Leonowicz *et al*., 1999). White rot fungi have a unique ability to produce ligninolytic enzymes to degrade lignin. Wood-rotting fungi are divided into three groups which are